MULTIPLE CYTOCHROMES b IN MYCOBACTERIUM PHLEI

Natalie S. Cohen, Eitan Bogin*, Taneaki Higashi** and Arnold F. Brodie

Department of Biochemistry
University of Southern California School of Medicine
Los Angeles, California 90033

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SUMMARY. Electron transport particles from M. phlei contain at least 3 different active forms of cytochrome b, one reduced by NADH, with a λ_{max} at 563 nm (b_N563), and the other two reduced by either succinate or NADH, with λ_{max} at 559 and 563 nm (b_S559) and (b_S563). Low temperature λ_{max} for cytochrome b reduction with NADH or succinate are described. During steady state only b_S563 was observed with succinate. In the presence of ATP, succinate reduced an increased amount of a b563. A branching of the NAD^+-linked pathway and a convergence at the level of cytochrome c is suggested, with only one branch accessible to succinate.

INTRODUCTION. Early studies of the electron transport pathways of phosphorylating membranes from Mycobacterium phlei (1) revealed that cytochromes c_1, c, and $a + a_3$ were almost completely reduced by either succinate or NAD^+-linked substrates while cytochrome b was only partially reduced by succinate or malate. Moreover, after completion of enzymatic reduction, even with all substrates combined, a relatively large amount of unreduced cytochrome b remained which was reducible by dithionite.

The discovery by Chance (2) of multiple forms of cytochrome b in mitochondria has led to many investigations of the occurrence, properties, and interrelationships of these components (3-9). Lanyi (10) described two b-type cytochromes in a halophilic bacterial electron transport chain and suggested that the cytochromes b were on separate pathways, converging at the c-type

* Present address: Institute of Veterinary Research, Biochemistry Division Beit Dagan, Israel.

**Present address: Department of Biochemistry, Institute for Cancer Research, Osaka University Medical School, Osaka, Japan.

cytochromes. This study describes the multiple cytochromes b found in M. phlei and their relationship to different electron transport pathways. A preliminary account of this work has appeared elsewhere (11).

MATERIALS AND METHODS. Electron transport particles (ETP) from Mycobacterium phlei were prepared as previously described (12). The washed ETP were resuspended for use in 10 mM $MgCl_2$. Protein was estimated by the method of Stadtman et al., (13). All spectral studies were performed on an Aminco spectrophotometer, Model DW-2.

RESULTS AND DISCUSSION. Spectrophotometric studies of cytochrome b are complicated since this component appears as a shoulder on the maximum (551 nm) of cytochrome c. Ascorbate and N,N,N',N'-tetramethyl-p-phenylenediamine (TPD) have been shown in M. phlei to reduce cytochromes c_1, c and $a + a_3$ (14). Thus the addition of ascorbate-TPD to reference and sample allows cytochrome b absorption peaks resulting from other substrate additions to be visualized.

The λ_{max} for cytochrome b reduced by succinate in M. phlei ETP were at 559, 528, and 430 nm (Fig. 1B). The peaks obtained with succinate were all rather broad and a shoulder could be seen on the α peak at about 563 nm. Difference spectra taken immediately after addition of substrate revealed that the first cytochrome b component reduced by succinate was at 563 nm (Fig. 2). The appearance of reduced cytochrome c is due to the higher rate of reduction of cytochrome c by succinate than by TPD since the latter concentration was rate limiting (14).

When NADH was used as a substrate (Fig. 1A), the cytochrome b absorption peaks were larger and had maxima different from those obtained with succinate (Fig. 1B). A combination of the two substrates reduced no more cytochrome b than did NADH alone, indicating that some of the cytochrome b reduced by NADH was identical to that reduced by succinate. An NADH reduced minus succinate reduced difference spectrum (Fig. 1C) revealed that

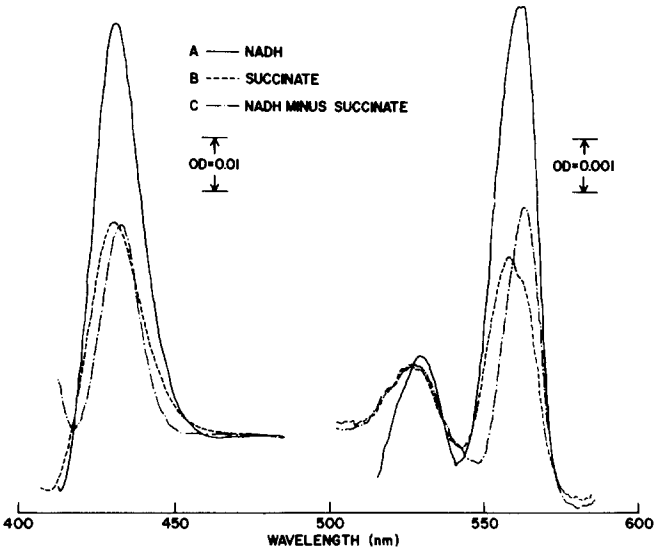

Fig. 1. Difference spectra of cytochrome b reduced by NADH or by succinate.

The reaction mixture consisted of 100 μmoles of HEPES-KOH buffer, pH 7.5, 15 μmoles of $MgCl_2$, 20 μmoles of ascorbate, 2 μmoles of TPD, ETP (6.85 mg), substrate, and water to a final volume of 3.1 ml. The optical path length was 1 cm. The spectra were taken after the cytochrome b was fully reduced in both cuvettes as determined by previous kinetic studies.

A : 5 μmoles of NADH in the sample cell.
B : 100 μmoles of succinate in the sample cell.
C : 5 μmoles of NADH in the sample and 100 μmoles of succinate in the reference cell.

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the λ_{max} for the cytochrome b reducible only by NADH were at 563, 530, and 432 nm. The λ_{max} listed here were confirmed using a Cary spectrophotometer, Model 14, with a 0 - .1 slide wire. Thus it appears that cytochrome b reduction occurs on two different pathways involving different types of cytochrome b: the NADH b (b_N563) and the succinate reduced components involving two b cytochromes, b_S563 and b_S559. It is of interest that although NADH can reduce the succinate cytochromes b, succinate cannot reduce the b_N563. It appears that the b_S559 and the b_S563 involve two forms of cytochrome b since during steady state the major absorption peak was at 563 nm.

In the presence of ATP, succinate reduced additional cytochrome b (Fig. 3A and B). The addition of ATP resulted in a slight red shift of all

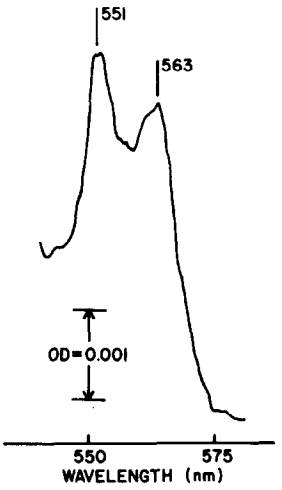

Fig. 2. Difference spectrum of cytochromes b and c reduced by succinate in the steady state.

The reaction mixture was the same as that described in Fig. 1, except that 7.18 mg of protein were used and the final volume was 3.0 ml. The spectrum was run 30 seconds after the addition of 100 μmoles of succinate to the sample cuvette.

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the cytochrome b absorption peaks. The λ_{max} of this ATP-dependent cytochrome are shown in a difference spectrum of succinate plus ATP reduced minus succinate reduced cytochrome b (Fig. 3C). The α peak was rather broad and the λ_{max} appeared to be at 563 nm and 433 nm. The presence of ATP decreased the amount of cytochrome b reduced by NADH.

Spectra of chemically reducible cytochrome b are shown in Fig. 4. Trace A indicates the total amount of chemically reducible cytochrome b in the ETP. Trace B shows that a substantial amount of cytochrome b could not be reduced enzymatically (dithionite reduced minus NADH reduced). Similar findings have been reported in yeast mitochondria by Sato et al., (9), who suggested that this pool of inactive cytochrome b might have been formed during preparation of the membranes.

Low temperature spectra of cytochrome b in ETP gave λ_{max} of 555, 528, and 428 nm, with prominent shoulders at 555 and 561 nm for the cyto-

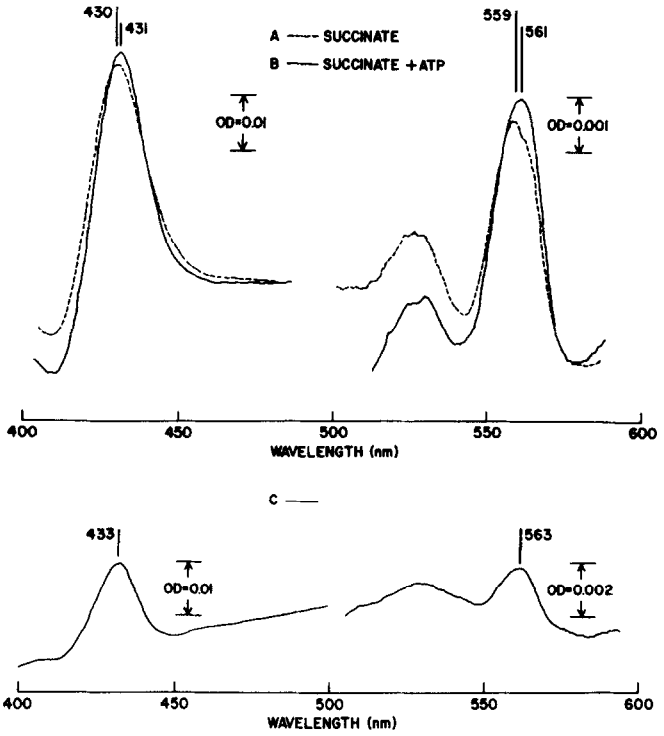

Fig. 3. Difference spectra of cytochrome b reduced by succinate and ATP.

The reaction mixture and conditions for traces A + B were the same as those described in Fig. 1.

A : 100 μmoles of succinate in the sample cell.
B : 5 μmoles of ATP and 100 μmoles of succinate in the sample cell.
C : The reaction mixture consisted of 100 μmoles of HEPES-KOH buffer, pH 7.5, 15 μmoles of $MgCl_2$, ETP (6.25 mg), substrate, and water to a final volume of 3.15 ml. In addition, the sample cell contained 5 μmoles of ATP and both cells received 100 μmoles of succinate.

chrome b reduced by succinate (Table I). Both the 555 absorption and the shoulder were increased in the presence of ATP. Spectra of succinate plus ATP reduced minus succinate reduced cytochrome b, exhibited λ_{max} for the ATP-dependent cytochrome b at 561, 530, and 431 nm, with small shoulders at about 556 and 428 nm. Cytochrome b_N563 (NADH reduced minus succinate reduced), exhibited low temperature λ_{max} at 561, 530, and 431 nm, with small shoulders at 557 and 428 nm. The spectrum of the total cytochrome b reduced

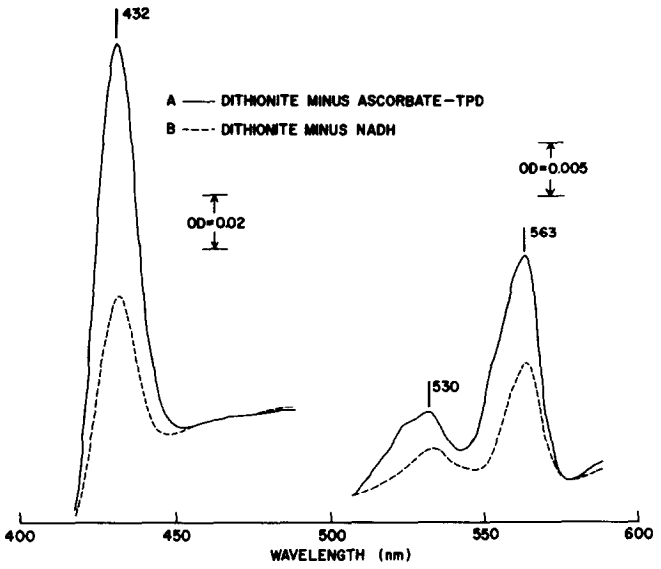

Fig. 4. Difference spectra of chemically reducible cytochrome b.

The reaction mixture consisted of 100 μmoles of HEPES-KOH buffer, pH 7.5, 15 μmoles of $MgCl_2$, and 6.42 mg of ETP in a final total volume of 3.1 ml.

A : Total chemically reducible cytochrome b. 20 μmoles of ascorbate and 2 μmoles of TPD were added to the reference cell. After cytochrome reduction was complete in the latter, few grains of solid dithionite were added to the sample. The spectrum was taken 4 min later.

B : Non-enzymatically reducible cytochrome b. The reference cell received 5 μmoles of NADH. After cytochrome b reduction was complete, solid dithionite was added to the sample cell and the spectrum was run 4 min later.

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by NADH had broad peaks with λ_{max} at 556, 561, 528, and one from 428 to 432 nm.

It is apparent that the ETP of M. phlei contain several b-type cytochromes which serve different pathways. A branching of the NAD^+-linked electron transport chain is involved since NADH can reduce the cytochromes on both pathways. The two pathways probably converge at cytochrome c_1, accounting for the incomplete sensitivity of NADH oxidation to 2-n-nonyl-hydroxyquinoline-N-oxide (NHQNO) which completely inhibits succinate oxidation, and the observed reduction of cytochrome c by NADH but not by succinate in the presence of NHQNO (14). Thus NHQNO inhibits on the cyto-

TABLE I

Low Temperature Absorption Maxima of Cytochromes b in M. phlei

Conditions				
Reference	Sample	α	β	γ
Ascorbate-TPD	Succinate + Ascorbate-TPD	555 (561S*)	528	428 (432S)
Ascorbate-TPD	Succinate + ATP + Ascorbate-TPD	555 (561S)	528-529	428-431
Succinate	Succinate + ATP	561 (556S)	530	431 (428S)
Succinate	NADH	561 (557S)	530	431 (428S)
Ascorbate-TPD	NADH + Ascorbate-TPD	556, 561 (broad)	528-529 (broad)	428-432 (broad)

The spectra were taken at the temperature of liquid nitrogen in an Aminco DW-2 spectrophotometer with a bandpass of 0.5 to 1.0 nm. The optimal path length was 2 mm. The reaction mixture consisted of 24 μmoles of HEPES-KOH buffer, pH 7.5, 3.3 μmoles of $MgCl_2$, 2.77 mg of ETP, substrates, and water to a final volume of 0.6 ml. The amounts of substrates used were 20 μmoles of succinate, 1 μmole of NADH, 10 μmoles of ascorbate and 1 μmole of TPD. One μmole of ATP was used. *S = shoulder.

chrome b_S pathway. The existence of two differentially accessible cytochromes b which have the same spectral characteristics (b_N563 and b_S563) might be due to some compartmentalization of the components and/or to differences in their oxidation reduction potentials. From the present data it is not clear whether the transfer of electrons from NADH to the cytochromes b on the succinate pathway occurs via the NADH cytochrome b or at a site prior to this. Lanyi (10) has suggested an interaction of NADH with the succinate pathway in a halophilic bacterium at the flavoprotein level.

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REFERENCES

1. Asano, A. and Brodie, A. F., J. Biol. Chem., 239, 4280 (1964).
2. Chance, B., J. Biol. Chem., 233, 735 (1958).

3. Dutton, P.L., Erecinska, M., Sato, N., Mukai, Y., Pring, M. and Wilson, D.F., Biochim. Biophys. Acta, 267, 15 (1972).
4. Slater, E.C., Lee, C.P., Berden, J.A. and Wegdam, H.J., Nature, 226, 1248 (1970).
5. Wikstrom, M.K.F., Biochim. Biophys. Acta, 253, 332 (1971).
6. Davis, K.A., Hatefi, Y., Poff, K.L. and Butler, W.L., Biochem. Biophys. Res. Commun., 46, 1984 (1972).
7. Yu, C.A., Yu, L. and King, T.E., Biochim. Biophys. Acta, 267, 300 (1972).
8. Norling, B., Nelson, B.D., Nordenbrand, K. and Ernster, L., Biochim. Biophys. Acta, 275, 18 (1972).
9. Sato, N., Ohnishi, T. and Chance, B., Biochim. Biophys. Acta, 275, 288 (1972).
10. Lanyi, J.K., J. Biol. Chem., 244, 2864 (1969).
11. Cohen, N.S., Hirata, H. and Brodie, A.F., Fed. Proc., 32, 1673Abs (1973).
12. Brodie, A.F., J. Biol. Chem., 234, 398 (1959).
13. Stadtman, E.R., Novelli, G.D. and Lipmann, F., J. Biol. Chem., 191, 365 (1951).
14. Orme, T.W., Revsin, B. and Brodie, A.F., Arch. Biochem. Biophys., 134, 172 (1969).